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(54) Title: INHIBITION OF NEOVASCULARIZATION USING VEGF-SPECIFIC OLIGONUCLEOTIDES

(57) Abstract

Disclosed are methods of reducing neovascularization and of treating various disorders associated with neovascularization. These methods include administering to a tissue or subject a synthetic oligonucleotide specific for vascular endothelial growth factor nucleic acid effective in inhibiting the expression of vascular endothelial growth factor.

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INHIBITION OF NEOVASCULARIZATION USING VEGF-SPECIFIC OLIGONUCLEOTIDES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of copending Patent Application Serial No. 08/098,942 entitled "Antisense Oligonucleotide Inhibition of Vascular Endothelial Growth Factor Expression, filed 27 July, 1993.

FUNDING

This invention was made with Government support under Grant No. RO1 EY0869 awarded by the National Institutes of Health. Thus, the Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

This invention relates to neovascularization. More specifically, this invention relates to treatment of disorders that are associated with neovascularization using oligonucleotides specific for vascular endothelial growth factor.

Neovascular diseases of the retina such as diabetic retinopathy, retinopathy of prematurity, and age-related macular degeneration are a major cause of blindness in the United States and the world, yet the biochemical events responsible for these processes have not been fully elucidated.

Diabetic retinopathy is the leading cause of blindness among working age adults (20-64) in the United States (Foster in *Harrison's Principles of Internal Medicine* (Isselbacher et al., ds.) McGraw-Hill, Inc., New York

(1994) pp. 1994-1995). During the course of diabetes mellitus, the retinal vessels undergo changes that result in not only leaky vessels but also vessel drop out resulting in retinal hypoxia. The effects of these complications are hemorrhaging, "cotton wool" spots, retinal infarcts, and neovascularization of the retina resulting in bleeding and retinal detachment. If left untreated, there is a 60% chance of visual loss. Classic treatment for proliferative diabetic retinopathy is panretinal laser photocoagulation (PRP). However, complications can occur from panretinal laser photocoagulation such as foveal burns, hemorrhaging, retinal detachment, and choroidal vessel growth. Furthermore, other untoward effects of this treatment are decreased peripheral vision, decreased night vision, and changes in color perception (*Am. J. Ophthalmol.* (1976) **81**:383-396; *Ophthalmol.* (1991) **98**:741-840).

Thus, there is a need for a more effective treatment for diabetic retinopathy.

Retinopathy of prematurity (ROP) is a common cause of blindness in children in the United States (Pierce et al. (1994) *Int. Ophth. Clinics* **34**:121-148). Premature babies are exposed to hyperoxic conditions after birth even without supplemental oxygen because the partial pressure of oxygen *in utero* is much lower than what is achieved when breathing normal room air. This relative hyperoxia is necessary for their survival yet can result in ROP. The blood vessels of the retina cease to develop into the peripheral retina resulting in ischemia and localized hypoxic conditions as the metabolic demands of the developing retina increase. The resulting hypoxia stimulates the subsequent neovascularization of the retina. This neovascularization usually regresses but can lead to irreversible vision loss. There are at least 10,000 new cases per year with a worldwide estimate of 10

million total cases. At present, there is no effective cure for ROP. Two therapeutic methods, cryotherapy and laser therapy, have been used but are not completely effective and themselves cause damage to the eye, resulting in a reduction of vision (Pierce et al. (1994) *Int. Ophth. Clinics* 34:121-148). Many other antiangiogenic compounds have been tested, but no inhibition in retinal neovascularization has been reported (Smith et al. (1994) *Invest. Ophthalmol. Vis. Sci.* 35:1442).

Thus, there is a need for an effective treatment for ROP.

Age related macular degeneration is one of the leading causes of blindness in older adults in the United States, and may account for up to 30% of all bilateral blindness among Caucasian Americans (Anonymous (1994) *Prevent Blindness America*). This disease is characterized by loss of central vision, usually in both eyes, due to damage to retinal pigment epithelial cells which provide physiological support to the light sensitive photoreceptor cells of the retina. In most cases there is currently no effective treatment. In approximately 20% of exudative cases that are diagnosed early, laser treatment can prevent further loss of vision; however, this effect is temporary (Bressler et al., *Principles and Practices of Ophthalmology* (eds. Albert and Jakobiac), W.B. Saunders Co., Philadelphia, PA) (1994) Vol. 2 pp. 834-852).

Thus, there is a need for a more effective and permanent treatment for age related macular degeneration.

Ocular neovascularization is also the underlying pathology in sickle cell retinopathy, neovascular glaucoma, retinal vein occlusion, and other hypoxic

diseases. These eye diseases as well as other pathological states associated with neovascularization (i.e., tumor growth, wound healing) appear to have hypoxia as a common factor (Knighton et al. (1983) *Science* **221**:1283-1285; Folkman et al. (1987) *Science* **235**:442-446; Klagsbrun et al. (1991) *Ann. Rev. Physiol.* **53**:217-239; Miller et al. (1993) *Principles and Practice of Ophthalmology*, W.B. Saunders, Philadelphia, pp. 760; and Aiello et al. (1994) *New Eng. J. Med.* **331**:1480-1487). Moreover, retinal neovascularization has been hypothesized to be the result of a "vasoformative factor" which is released by the retina in response to hypoxia (Michaelson (1948) *Trans. Ophthalmol. Soc. U. K.* **68**:137-180; and Ashton et al. (1954) *Br. J. Ophthalmol.* **38**:397-432). Recent experimental data show a high correlation between vascular endothelial growth factor expression and retinal neovascularization (Aiello et al. (1994) *New Eng. J. Med.* **331**:1480-1487). Furthermore, elevated levels of vascular endothelial growth factor have recently been found in vitreous from patients with diabetes (Aiello et al., *ibid.*). Thus, this cytokine/growth factor may play an important role in neovascularization-related disease.

Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) is an endothelial cell-specific mitogen which has recently been shown to be stimulated by hypoxia and required for tumor angiogenesis (Senger et al. (1986) *Cancer Res.* **46**:5629-5632; Kim et al. (1993) *Nature* **362**:841-844; Schweiki et al. (1992) *Nature* **359**:843-845; Plate et al. (1992) *Nature* **359**:845-848). It is a 34-43 kDa (with the predominant species at about 45 kDa) dimeric, disulfide-linked glycoprotein synthesized and secreted by a variety of tumor and normal cells. In addition, cultured human retinal cells such as pigment epithelial cells and pericytes have been demonstrated to

secrete VEGF and to increase VEGF gene expression in response to hypoxia (Adamis et al. (1993) *Biochem. Biophys. Res. Commun.* **193**:631-638; Plouet et al. (1992) *Invest. Ophthalmol. Vis. Sci.* **34**:900; Adamis et al. (1993) *Invest. Ophthalmol. Vis. Sci.* **34**:1440; Aiello et al. (1994) *Invest. Ophthalmol. Vis. Sci.* **35**:1868; Simorre-Pinatel et al. (1994) *Invest. Ophthalmol. Vis. Sci.* **35**:3393-3400). In contrast, VEGF in normal tissues is relatively low. Thus, VEGF appears to play a principle role in many pathological states and processes related to neovascularization. Regulation of VEGF expression in tissues affected by the various conditions described above could therefore be key in treatment or preventative therapies associated with hypoxia.

SUMMARY OF THE INVENTION

It is known that cells affected by hypoxia express VEGF. It has now been discovered that synthetic oligonucleotides specific for the mRNA for VEGF can inhibit hypoxia-induced neovascularization. This information has been exploited to develop the present invention which includes methods of reducing neovascularization and of treating disorders and diseases related to neovascularization. As used herein, the term "neovascularization" refers to the growth of blood vessels and capillaries.

In the methods of the invention, an amount of a synthetic oligonucleotide specific for vascular endothelial growth factor nucleic acid and effective in inhibiting the expression of vascular endothelial growth factor is administered to a neovascularized tissue. This tissue may be a culture or may be part or the whole body of an animal such as a human or other mammal.

As used herein, the term "synthetic oligonucleotide" refers to chemically synthesized polymers of nucleotides covalently attached via at least one 5' to 3' internucleotide linkage. In some embodiments, these oligonucleotides contain at least one deoxyribonucleotide, ribonucleotide, or both deoxyribonucleotides and ribonucleotides. In another embodiment, the synthetic oligonucleotides used in the methods of the invention are from about 14 to about 28 nucleotides in length. In preferred embodiments, these oligonucleotides contain from about 15 to about 25 nucleotides.

In some embodiments, the oligonucleotides may also be modified in a number of ways without compromising their ability to hybridize to nucleotide sequences contained within the mRNA for VEGF. The term "modified oligonucleotide" as used herein describes an oligonucleotide in which at least two of its nucleotides are covalently linked via a synthetic linkage, i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide in which the 5' nucleotide phosphate has been replaced with any number of chemical groups.

In some embodiments, at least one internucleotide linkage of the oligonucleotide is an alkylphosphonate, phosphorothioate, phosphorodithioate, phosphate ester, alkylphosphonothioate, phosphoramidate, carbamate, carbonate, phosphate triester, acetamidate, and/or carboxymethyl ester.

The term "modified oligonucleotide" also encompasses oligonucleotides with a modified base and/or sugar. In addition, unoxidized or partially oxidized oligonucleotides having a substitution in one nonbridging oxygen per nucleotide in the molecule are considered to

be modified oligonucleotides. Also considered as modified oligonucleotides are oligonucleotides having nuclease resistance-conferring bulky substituents at their 3' and/or 5' end(s) and/or various other structural modifications not found *in vivo* without human intervention. Other modifications include those which are internal or are at the end(s) of the oligonucleotide molecule and include additions to the molecule of the internucleoside phosphate linkages, and terminal ribose, deoxyribose and phosphate modifications which cleave, or crosslink to the opposite chains or to associated enzymes or other proteins which bind to the genome.

A method of treating retinopathy of prematurity (ROP) is provided. This method comprises the step of administering to a subject afflicted with ROP a therapeutic amount of an oligonucleotide specific for vascular endothelial growth factor nucleic acid and effective in inhibiting the expression of vascular endothelial growth factor in the retina.

In another aspect of the invention, a method of treating diabetic retinopathy is provided. This method includes administering to a subject afflicted with diabetic retinopathy a therapeutic amount of an oligonucleotide specific for vascular endothelial growth factor nucleic acid and effective in inhibiting the expression of VEGF in the retina.

In yet another aspect of the invention, a method of treating age-related macular degeneration (ARMD) is provided, which includes comprising the step of administering to a subject afflicted with ARMD a therapeutic amount of an oligonucleotide specific for vascular endothelial growth factor nucleic acid effective in inhibiting the expression of VEGF in the retina.

In some preferred embodiments of the methods of the invention described above, the oligonucleotide is administered locally (e.g., intraocularly or interlesionally) and/or systemically. The term "local administration" refers to delivery to a defined area or region of the body, while the term "systemic administration is meant to encompass delivery to the whole organism by oral ingestine, or by intramuscular, intravenous, subcutaneous, or intraperitoneal injection.

Another aspect of the invention includes pharmaceutical compositions capable of inhibiting neovascularization and thus are useful in the methods of the invention. These compositions include a synthetic oligonucleotide which specifically inhibits the expression of vascular endothelial growth factor and a physiologically and/or pharmaceutically acceptable carrier.

The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The term "physiologically acceptable" refers to a non-toxic material that is compatible with a biological system such as a cell, cell culture, tissue, or organism.

Another aspect of the invention is assessment of the role of VEGF in neovascularization associated with disease states.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

FIG. 1 is a diagrammatic representation of the murine model for retinal neovascularization;

FIG. 2 is a graphic representation of the ability of oligonucleotides of the invention to inhibit neovascularization during retinopathy of prematurity;

FIG. 3 is a diagrammatic representation of the ELISA used to test the ability of human VEGF-specific oligonucleotides to inhibit the expression of VEGF;

FIG. 4 is a graphic representation of the results of an ELISA demonstrating the reduction in the expression of VEGF in human cells in the presence of human VEGF-specific oligonucleotides of the invention; and

FIG. 5 is a graphic representation of the results of a Northern blot demonstrating the reduction in the expression of VEGF by human cells in the presence of varying concentrations of human VEGF-specific oligonucleotides of the invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. The issued U.S. patents, allowed applications, and references cited herein are hereby incorporated by reference.

The present invention provides synthetic antisense oligonucleotides specific for VEGF nucleic acid which are useful in treating diseases and disorders associated with neovascularization including retinal neovascularization.

Antisense oligonucleotide technology provides a novel approach to the inhibition of gene expression (see generally, Agrawal (1992) *Trends in Biotech.* **10**:152; Wagner (1994) *Nature* **372**:333-335; and Stein et al. (1993) *Science* **261**:1004-1012). By binding to the complementary nucleic acid sequence (the sense strand), antisense oligonucleotide are able to inhibit splicing and translation of RNA. In this way, antisense oligonucleotides are able to inhibit protein expression. Antisense oligonucleotides have also been shown to bind to genomic DNA, forming a triplex, and inhibit transcription. Furthermore, a 17mer base sequence statistically occurs only once in the human genome, and thus extremely precise targeting of specific sequences is possible with such antisense oligonucleotides.

It has been determined that the VEGF coding region is comprised of eight axons (Tischer et al. (1994) *J. Biol. Chem.* **266**:11947-11954). Three VEGF transcripts, 121, 165, and 189 amino acids long, have been observed, suggesting that an alternative splicing mechanism is involved (Leung et al. (1989) *Science* **246**:1306-1309; Tischer et al. (1991) *J. Biol. Chem.* **266**:11947-11954). More recently, a fourth

VEGF transcript was discovered which has a length encoding 206 amino acids (Houck et al. (1991) *Mol. Endocrinol.* 5:1806-1814). Transcripts analogous to the 121 and 165 amino acid polypeptides have been identified in the bovine system (Leung et al. (1989) *Science* 246:1306-1309), and the transcript corresponding to the 165 amino acid transcript have also been identified in the rodent system (Conn et al. (1990) *Proc. Natl. Acad. Sci. (USA)* 87:1323-1327); Senger et al. (1990) *Cancer Res.* 50:1774-1778; Claffey et al. (1992) *J. Biol. Chem.* 267:16317-16322). Nucleic acid sequences encoding three forms of VEGF have also been reported in humans (Tischer et al. (1991) *J. Biol. Chem.* 266:11947-11954), and comparisons between the human and the murine VEGF have revealed greater than 85% interspecies conservation (Claffey et al. (1992) *J. Biol. Chem.* 267:16317-16322).

The oligonucleotides of the invention are directed to any portion of the VEGF nucleic acid sequence that effectively acts as a target for inhibiting VEGF expression. The sequence of the gene encoding VEGF has been reported in mice (Claffey et al., *ibid.*) and for humans (Tischer et al., *ibid.*). These targeted regions of the VEGF gene include any portions of the known exons. In addition, exon-intron boundaries are potentially useful targets for antisense inhibition of VEGF expression.

The nucleotide sequences of some representative, non-limiting oligonucleotides specific for human VEGF are listed below in TABLE 1.

TABLE 1

OLIGO	TARGETED SITE	SEQUENCE (AS)	SEQ ID NO:
H-1	21-2	5'-CGCCGGGCCGCCAGCACACT-3'	1
H-1R	21-2	5'-CGCCGGGCCGCCAGCACACU-3'	2
H-1A	16-2	5'-GGCCGCCAGCACACT-3'	3
H-1B	26-2	5'-GCTCGCGCCGGGCCGCCAGCACACT-3'	4
H-2	76-57	5'-CAAGACAGCAGAAAGTTCAT-3'	5
H-3	80-62	5'-CACCCAAGACAGCAGAAAG-3'	6
H-3A	75-62	5'-CACCCAAGACAGCAG-3'	7
H-3B	86-62	5'-CCAATGCACCCAAGACAGCAGAAAG-3'	8
H-4	64-45	5'-AAGTTCATGGTTTCGGAGGC-3'	10
H-5	62-43	5'-GTTCATGGTTTCGGAGGCCC-3'	11
H-6	138-119	5'-GTGCAGCCTGGGACCACTTG-3'	12
H-7	628-609	5'-CGCCTCGGCTTGTCACATCT-3'	13
H-8	648-629	5'-CTTCCTCCTGCCCGGCTCAC-3'	14
H-8R	648-629	5'-CUUCCUCCUGCCCGGCUCAC-3'	15
H-8A	643-629	5'-CTTCCTCCTGCCCGG-3'	16
H-8B	653-629	5'-GGCTCCTTCCTCCTGCCCGGCTCAC-3'	17
H-9	798-779	5'-GTCTCCTCTTCCTTCATTTC-3'	18
H-9A	793-779	5'-GTCTCCTCTTCCTTC-3'	19
H-9B	803-779	5'-GCAGAGTCTCCTCTTCCTTCATTTC-3'	20
H-10	822-803	5'-CGGACCCAAAGTGCTCTGCG-3'	21
H-10A	817-803	5'-CCAAAGTGCTCTGCG-3'	22
H-10B	827-803	5'-CCCTCCGGACCCAAAGTGCTCTGCG-3'	23
H-11	E1-I1	5'-GGGCACGACCGCTTACCTTG-3'	24
H-12	I1-E2	5'-GGGACCACTGAGGACAGAAA-3'	25
H-13	I2-E3	5'-CACCACTGCATGAGAGGCGA-3'	26
H-14	E3-I3	5'-TCCCAAAGATGCCCACCTGC-3'	27
H-15	I3-E4	5'-CGCATAATCTGGAAAGGAAG-3'	28
H-17	59-40	5'-CATGGTTTCGGAGGCCCGAC-3'	30
H-17B	59-40	5'-CAUGGTTUCGGAGGCCCGAC-3'	31
H-18	61-42	5'-TTCATGGTTTCGGAGGCCCG-3'	32
E1/I1	E1/I1	5'-GACCGCTTACCTTGGCATGG-3'	33
I1/E2	I1/E2	5'-CCTGGGACCACTGAGGACAG-3'	34
E2/I2	E2/I2	5'-GGGACTCACCTTCGTGATGA-3'	35
I2/E3	I2/E3	5'-GAACTTCACCACTGCATGAG-3'	36
E3/I3	E3/I3	5'-TCCCAAAGATGCCCACCTGC-3'	37
I3/E4	I3/E4	5'-GCATAATCTGGAAAGGAAGG-3'	38
E4/I4	E4/I4	5'-ACATCCTCACCTGCATTCAC-3'	39
E4/I4B	E4/I4	5'-ACATCCUCACCTGCAUUCAC-3'	40
I4/E5	I4/E5	5'-TTTCTTTGGTCTGCAATGGG-3'	41
E5/I5	E5/I5	5'-GGCCACTTACTTTTCTTGTC-3'	42
I5/E7	I5/E7	5'-CACAGGGACTGGAAAATAAA-3'	43
E7/I7	E7/I7	5'-GGGAACCAACCTGCAAGTAC-3'	44
I7/E8	I7/E8	5'-GTCACATCTGAGGGAAATGG-3'	45
VH	641-621	5'-CTGCCCGGCTCACCGCCTCGG-3	46
H-19	56-38	5'-GGTTTCGGAGGCCCGACCG-3'	50

With the published nucleic acid sequences and this disclosure provided, those of skill in the art will be able to identify, with without undue experimentation, other antisense nucleic acid sequences that inhibit VEGF expression. For example, other sequences targeted specifically to human VEGF nucleic acid can be selected based on their ability to be cleaved by RNase H.

The oligonucleotides of the invention are composed of ribonucleotides, deoxyribonucleo-tides, or a combination of both, with the 5′ end of one nucleotide and the 3′ end of another nucleotide being covalently linked. These oligonucleotides are at least 14 nucleotides in length, but are preferably 15 to 28 nucleotides long, with 15 to 25mers being the most common.

These oligonucleotides can be prepared by the art recognized methods such as phosphoramidate or H-phosphonate chemistry which can be carried out manually or by an automated synthesizer as described in Uhlmann et al. (*Chem. Rev.* (1990) **90**:534-583).

The oligonucleotides of the invention may also be modified in a number of ways without compromising their ability to hybridize to VEGF mRNA. For example, the oligonucleotides may contain other than phosphodiester internucleotide linkages between the 5′ end of one nucleotide and the 3′ end of another nucleotide in which the 5′ nucleotide phosphodiester linkage has been replaced with any number of chemical groups. Examples of such chemical groups include alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters, carbonates, and

phosphate triesters. Oligonucleotides with these linkages can be prepared according to known methods (see, e.g., Uhlmann et al. (1990) *Chem. Rev.* 90:543-583).

Other modifications include those which are internal or at the end(s) of the oligonucleotide molecule and include additions to the molecule of the internucleoside phosphate linkages, such as cholesteryl or diamine compounds with varying numbers of carbon residues between the amino groups and terminal ribose, deoxyribose and phosphate modifications which cleave, or crosslink to the opposite chains or to associated enzymes or other proteins which bind to the genome. Examples of such modified oligonucleotides include oligonucleotides with a modified base and/or sugar such as 2'-O-alkylated ribose, arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position). Other modified oligonucleotides are capped with a nuclease resistance-conferring bulky substituent at their 3' and/or 5' end(s), or have a substitution in one nonbridging oxygen per nucleotide. Such modifications can be at some or all of the internucleoside linkages, as well as at either or both ends of the oligonucleotide and/or in the interior of the molecule.

The preparation of these modified oligonucleotides is well known in the art (reviewed in Agrawal et al. (1992) *Trends Biotechnol.* 10:152-158). For example, nucleotides can be covalently linked using art-recognized techniques such as

phosphoramidate, H-phosphonate chemistry, or methylphosphoramidate chemistry (*see, e.g.*, Uhlmann et al. (1990) *Chem. Rev.* **90**:543-584; Agrawal et al. (1987) *Tetrahedron. Lett.* **28**:(31):3539-3542); Caruthers et al. (1987) *Meth. Enzymol.* **154**:287-313; U.S. Patent 5,149,798). Oligomeric phosphorothioate analogs can be prepared using methods well known in the field such as methoxyphosphoramidite (*see, e.g.*, Agrawal et al. (1988) *Proc. Natl. Acad. Sci. (USA)* **85**:7079-7083) or H-phosphonate (*see, e.g.*, Froehler (1986) *Tetrahedron Lett.* **27**:5575-5578) chemistry. The synthetic methods described in Bergot et al. (*J. Chromatog.* (1992) **559**:35-42) can also be used.

The synthetic antisense oligonucleotides of the invention in the form of a therapeutic formulation are useful in treating diseases, and disorders, and conditions associated with neovascularization including, but not limited to, retinal neovascularization, tumor growth, and wound healing.

The synthetic oligonucleotides of the invention may be used as part of a pharmaceutical composition when combined with a physiologically and/or pharmaceutically acceptable carrier. The characteristics of the carrier will depend on the route of administration. Such a composition may contain, in addition to the synthetic oligonucleotide and carrier, diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art. The pharmaceutical composition of the invention may also contain other active factors and/or agents which enhance inhibition of VEGF expression or which will reduce neovascularization. For example, combinations of synthetic oligonucleotides, each of which is

directed to different regions of the VEGF mRNA, may be used in the pharmaceutical compositions of the invention. The pharmaceutical composition of the invention may further contain nucleotide analogs such as azidothymidine, dideoxycytidine, dideosyinosine, and the like. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect with the synthetic oligonucleotide of the invention, or to minimize side-effects caused by the synthetic oligonucleotide of the invention. Conversely, the synthetic oligonucleotide of the invention may be included in formulations of a particular anti-VEGF or anti-neovascularization factor and/or agent to minimize side effects of the anti-VEGF factor and/or agent.

The pharmaceutical composition of the invention may be in the form of a liposome in which the synthetic oligonucleotides of the invention is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers which are in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. One particularly useful lipid carrier is lipofectin. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Patent No. 4,235,871; U.S. Patent No. 4,501,728; U.S. Patent No. 4,837,028; and U.S. Patent No. 4,737,323. The pharmaceutical composition of the invention may further include compounds such as cyclodextrins and the like which enhance delivery of

oligonucleotides into cells, as described by Zhao et al. (in press), or slow release polymers.

As used herein, the term "therapeutically effective amount" means the total amount of each active component of the pharmaceutical composition or method that is sufficient to show a meaningful patient benefit, i.e., healing of chronic conditions characterized by neovascularization or a reduction in neovascularization, itself, or in an increase in rate of healing of such conditions. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

In practicing the method of treatment or use of the present invention, a therapeutically effective amount of one or more of the synthetic oligonucleotide of the invention is administered to a subject afflicted with a disease or disorder related to neovascularization, or to a tissue which has been neovascularized. The synthetic oligonucleotide of the invention may be administered in accordance with the method of the invention either alone of in combination with other known therapies for neovascularization. When co-administered with one or more other therapies, the synthetic oligonucleotide of the invention may be administered either simultaneously with the other treatment(s), or sequentially. If administered sequentially, the attending physician will decide on the appropriate sequence of administering the

synthetic oligonucleotide of the invention in combination with the other therapy.

Administration of the synthetic oligonucleotide of the invention used in the pharmaceutical composition or to practice the method of the present invention can be carried out in a variety of conventional ways, such as intraocular, oral ingestion, inhalation, or cutaneous, subcutaneous, intramuscular, or intravenous injection.

When a therapeutically effective amount of synthetic oligonucleotide of the invention is administered orally, the synthetic oligonucleotide will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% synthetic oligonucleotide and preferably from about 25 to 90% synthetic oligonucleotide. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by weight of the synthetic oligonucleotide and preferably from about 1 to 50% synthetic oligonucleotide.

When a therapeutically effective amount of synthetic oligonucleotide of the invention is administered by intravenous, subcutaneous, intramuscular, intraocular, or intraperitoneal injection, the synthetic oligonucleotide will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, subcutaneous, intramuscular, intraperitoneal, or intraocular injection should contain, in addition to the synthetic oligonucleotide, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art.

The amount of synthetic oligonucleotide in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patent has undergone. Ultimately, the attending physician will decide the amount of synthetic oligonucleotide with which to treat each individual patient. Initially, the attending physician will administer low doses of the synthetic oligonucleotide and observe the patient's response. Larger doses of synthetic oligonucleotide may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further. It is

contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain about 10 μg to about 20 mg of synthetic oligonucleotide per kg body or organ weight.

The duration of intravenous therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

Some diseases lend themselves to acute treatment while others require to longer term therapy. Proliferative retinopathy can reach a threshold in a matter of days as seen in ROP, some cases of diabetic retinopathy, and neovascular glaucoma. Premature infants are at risk for neovascularization around what would be 35 weeks gestation, a few weeks after birth, and will remain at risk for a short period of time until the retina becomes vascularized. Diabetic retinopathy can be acute but may also smolder in the proliferative phase for considerably longer. Diabetic retinopathy will eventually become quiescent as the vasoproliferative signal diminishes with neovascularization or destruction of the retina.

Both acute and long term intervention in retinal disease are worthy goals. Intravitreal injections of oligonucleotides against VEGF can be an effective means of inhibiting retinal neovascularization in an acute situation. However

for long term therapy over a period of years, systemic delivery (intraperitoneal, intramuscular, subcutaneous, intravenous) either with carriers such as saline, slow release polymers, or liposomes should be considered.

In some cases of chronic neovascular disease, systemic administration of oligonucleotides may be preferable. Since the disease process concerns vessels which are abnormal and leaky, the problem of passage through the blood brain barrier may not be a problem. Therefore, systemic delivery may prove efficacious. The frequency of injections is from continuous infusion to once a month, depending on the disease process and the biological half life of the oligonucleotides.

In addition to inhibiting neovascularization *in vivo*, antisense oligonucleotides specific for VEGF are useful in determining the role of this cytokine in processes where neovascularization is involved. For example, this technology is useful in *in vitro* systems which mimic blood vessel formation and permeability, and in *in vivo* system models of neovascularization, such as the murine model described below.

A murine model of oxygen-induced retinal neovascularization has been established which occurs in 100% of treated animals and is quantifiable (Smith et al. (1994) *Invest. Ophthalmol. Vis. Sci.* 35:101-111). Using this model, a correlation has been determined between increasing expression of VEGF message and the onset of retinal neovascularization in the inner nuclear and ganglion cell layers (i.e., in Müller cells) (Pierce et al. (1995) *Proc. Natl. Acad.*

Sci. (USA) (in press). This result has been confirmed by Northern blot and *in situ* hybridization analysis of whole retinas at different time points during the development of neovascularization (Pierce et al., *ibid.*).

That VEGF plays a role in retinal neovascularization has been shown using the murine model of neovascularization described above. Three independent experiments were performed using antisense oligonucleotides specific for VEGF (JG-3 (SEQ ID NO:47), JG-4, (SEQ ID NO:48), and Vm (SEQ ID NO:46), and a corresponding sense oligonucleotide (V2 (SEQ ID NO:49). These oligonucleotides were designed using the known nucleic sequence of murine VEGF (Claffee et al. (1992) *J. Biol. Chem.* **267**:16317-16322). The sequence of the Vm oligonucleotide (SEQ ID NO:46) is targeted to the sequence surrounding the translational TGA stop site (TGA). The sequence of JG-4 (SEQ ID NO:48) is targeted to the sequence 5' to and containing the ATG of the translational start site of the murine VEGF molecule. The sequence of JG-3 (SEQ ID NO:47) is targeted to the 5' untranslated region, and the V2 sense sequence is targeted to the sequence surrounding the translational start site (ATG).

A compilation of the results of these experiments is presented in FIG. 2. These results indicate that Vm (SEQ ID NO:46) antisense oligonucleotide significantly reduces retinal neovascularization when compared with both untreated and sense oligonucleotide V2, (SEQ ID NO:49) controls. JG-3 (SEQ ID NO:47) and JG-4 (SEQ ID NO:47) show significant activity when compared against untreated eyes. The sense control oligonucleotide V2 (SEQ ID NO:49) does not show any

significant activity when compared with untreated eyes.

In the studies described above, the human VEGF antisense oligonucleotide which corresponds to murine JG-3 is H-1 (SEQ ID NO:1), which is targeted to the 5' untranslated region; that which corresponds to murine JG-4 is H-17 (SEQ ID NO:30), which is targeted to the sequence 5' to and containing the ATG of the translational start site of the human VEGF molecule; and that which corresponds to the murine Vm gene is VH (SEQ ID NO:46), which is targeted to sequences surrounding the translational stop site (TGA) of the human VEGF molecule. These antisense oligonucleotides of the invention are expected to inhibit VEGF expression in human cells in much the same way as the murine antisense oligonucleotides inhibit expression of VEGF in mouse cells.

Human VEGF antisense sequences corresponding to other murine sequences are also known. For example, human oligonucleotide H-6 (SEQ ID NO:12) corresponds to a region spanning murine sequences JG-6 (SEQ ID NO:52) and JG-7 (SEQ ID NO:53), and human oligonucleotide H-2 (SEQ ID NO:5) is in the same region as murine sequence JG-5 (SEQ ID NO:51). It is likely that these sequences have a similar effect on inhibition of VEGF expression and hence on controlling neovascularization.

There are several methods by which the effects of antisense oligonucleotides on VEGF expression and neovascularization can be monitored. One way is a capture ELISA developed for quantifying human VEGF protein expressed by cells. Using this assay, it has been determined that an antisense

phosphorothioate oligonucleotide H-3 (SEQ ID NO:6) targeted to a sequence just 3' to the translational start site can inhibit the hypoxic induction of VEGF expression in a sequence-specific manner, compared with random (R) and sense (H-16, SEQ ID NO:29) controls), as shown in FIG. 4. This inhibition is reproducible and in this *in vitro* system appears to be lipid carrier-specific and antisense-specific as only antisense oligonucleotide H-3 (SEQ ID NO:6) in the presence of lipofectin (a lipid carrier), and not lipofectamine (another lipid carrier), results in inhibition of VEGF protein expression.

At the RNA level, Northern blots (Sambrook et al. (1989) *Molecular Cloning; a Laboratory Manual*, Cold Spring Harbor Laboratory Press, NY, Vol. 1, pp. 7.38; Arcellana-Panlilio et al. (1993) *Meth. Enz.* **225**:303-328) can be performed to determine the extent that oligonucleotides of the invention inhibit the expression of VEGF mRNA. For example, as shown in FIG. 5, a histogram representing Northern blot analysis demonstrates a decrease in VEGF RNA levels in culture human cells treated with antisense oligonucleotide H-3 (SEQ ID NO:6), while there is only a minimal change in VEGF RNA levels in samples treated with sense control H-16 (SEQ ID NO:29).

In addition, bioactivity can be determined by several methods, including the Miles vessel permeability assay (Miles and Miles (1952) *J. Physiol. (Lond.)* **118**:228), which measures vessel permeability, endothelial cell mitogenicity, which measures cell growth, and intracellular calcium release in endothelial cells (see, e.g., Brock and Capasso (1988) *J. Cell. Physiol.* **136**:54), which measures the

release of calcium in response to VEGF binding to its receptor on endothelial cells.

The following examples illustrate the preferred modes of making and practicing the present invention, but are not meant to limit the scope of the invention since alternative methods may be utilized to obtain similar results.

EXAMPLE 1
PREPARATION OF VEGF-SPECIFIC OLIGONUCLEOTIDES

Human VEGF cDNA is transcribed *in vitro* using an *in vitro* eukaryotic transcription kit (Stratagene, La Jolla, CA). The RNA is labelled with ^{32}P using T-4 polynucleotide kinase as described by (Sambrook et al. (1989) *Molecular Cloning; a Laboratory Manual*, Cold Spring Harbor Laboratory Press, NY, Vol. 1, pp. 5.71). The labelled RNA is incubated in the presence of a randomer 20mer library and RNAse H, an enzyme which cleaves RNA-DNA duplexes (Boehringer Mannheim, Indianapolis, IN). Cleavage patterns are analyzed on a 6% polyacrylamide urea gel. The specific location of the cleaved fragments is determined using a human VEGF sequence ladder (Sequenase Kit, United States Biochemical, Cleveland, OH).

EXAMPLE 2
ANIMAL MODEL OF RETINAL NEOVASCULARIZATION

A. Preparation of Oligonucleotides

Synthesis of the following oligonucleotides: JG-3 (SEQ ID NO:47), JG-4 (SEQ ID NO:48), Vm (SEQ ID NO:46), and V2 (SEQ ID NO:49), was performed on a Pharmacia Gene Assembler series synthesizer using the phosphoramidite procedure (see, e.g., Uhlmann

et al. (*Chem. Rev.* (1990) **90**:534-583). Following assembly and deprotection, oligonucleotides were ethanol precipitated twice, dried, and suspended in phosphate-buffered saline (PBS) at the desired concentration.

The purity of these oligonucleotides was tested by capillary gel electrophoreses and ion exchange HPLC. Endotoxin levels in the oligonucleotide preparation was determined using the Luminous Amebocyte Assay (Bang (1953) *Biol. Bull.* (Woods Hole, MA) **105**:361-362).

B. Preparation of Animal Model

Seven day postnatal mice (P7, C57b1/6J, (Children's Hospital Breeding Facilities, Boston, MA) were exposed to 5 days of hyperoxic conditions (75 +/- 2%) oxygen in a sealed incubator connected to a Bird 3-M oxygen blender (flow rate: 1.5 liters/minute; Bird, Palm Springs, CA). The oxygen concentration was monitored by means of an oxygen analyzer (Beckman, Model D2, Irvine, CA). After 5 days (P12), the mice were returned to room air. Maximal retinal neovascularization was observed 5 days after return to room air (P17). After P21, the level of retinal neovascularization was just beginning to regress.

C. Treatment

After mice had been removed from oxygen, antisense oligonucleotides were injected into the vitreous with a Hamilton syringe and a 33 gauge needle (Hamilton Company, Reno, NV). The animals were anesthetized for the procedure with Avertin ip. The mice were given a single injection of antisense

oligonucleotides (or sense or non-sense controls) at P12 achieving a final concentration of approximately 30 μM. The animals were sacrificed at P17 with tribromoethanol ip (0.1 ml/g body weight) and cervical dislocation.

D. Microscopy

The eyes were enucleated, fixed in 4% paraformaldehyde, and embedded in paraffin. Serial sections of the whole eyes were cut sagittally, through the cornea, and parallel to the optic nerve. The sections were stained with hematoxylin and periodic acid-Schiff (PAS) stain. The extent of neovascularization in the treated eyes was determined by counting endothelial cell nuclei extending past the internal limiting membrane into the vitreous. Nuclei from new vessels and vessel profiles could be distinguished from other structures in the retina and counted in cross-section with light microscopy. Additional eyes were sectioned and examined by *in situ* hybridization to a VEGF probe.

To examine the retinal vasculature using fluorescein-dextran, the mice were perfused with a 50 mg/ml solution of high molecular weight fluorescein-dextran (Sigma Chemical Company, St. Louis, MO) in 4% paraformaldehyde. The eyes were enucleated, fixed in paraformaldehyde, and flat-mounted with glycerol-gelatin. The flat-mounted retinas were viewed and photographed by fluorescence microscopy using an Olympus BX60 fluorescence microscope (Olympus America Corp., Bellingham, MA).

EXAMPLE 3
RETINOPATHY OF PREMATURITY

A. Preparation of Oligonucleotides

VEGF specific oligonucleotides are synthesized as described in EXAMPLE 2A above. Sterile and endotoxin-free oligonucleotides are diluted in Balanced Salt Solution (BSS, Alcon, Fort Worth, TX) so as to have the same pH and electrolyte concentration as the aqueous or vitreous of the eye. Emalphor EC620 (2.5%, GAF Corp.) (Bursell et al. (1993) *J. Clin. Invest.* 92:2872-2876), a petroleum product, is added to change viscosity and aid in delivery properties. Doses to achieve intravitreal concentrations ranging from 0.1 μM - 100 μM are administered depending on the severity of the retinal/ocular neovascularization. The volume delivered is between 1 μl and 1 ml depending on the volume of the eye.

B. ROP Patient Profile

The patient treated is a premature, 34 week post-conception Caucasian female weighing less than 1,000 grams at birth and is respirator-dependent. The patient has bilateral stage 3+ disease with 11 clock hours of neovascularization in each eye. There is hemorrhaging in one eye, and both eyes have reached "threshold" according to the international classification (i.e., each eye has >50% chance of going on to retinal detachment). Extraretinal fibrovascular proliferation is found in both eyes.

C. Treatment

The intubated patient is anesthetized with fluorane. The face and eyes are prepared with a betadine scrub and draped in the usual sterile fashion. The sterile drug with vehicle is injected with a 33 gauge needle on a sterile syringe at the posterior limbus (pars plana) through full thickness sclera into the vitreous. No closing suture is required unless there is leakage. Antibiotic drops containing gentamicin or erythromycin ointment is applied to the surface of the globe in the palpebral fissure several times per day until there is complete wound closure. The frequency of injection ranges from every other day to once every 6 months or less, depending on the severity of the disease process, the degree of intraocular inflammation, the character of the vehicle (i.e., slow release characteristics), the degree of inhibition of the neovascularization and the tolerance of the eye to injections. Short and long term follow-up check-ups for possible retinal detachment from the neovascular disease as well as from the injections are necessary.

D. Monitoring of Progress

The eye upon dilation is monitored for signs of inflammation, infection, and resolution of neovascularization by both a direct and a indirect ophthalmoscope to view the retina and fundus. A slit lamp exam is used in some cases of anterior segment disease. Positive response to treatment includes fewer neovascular tufts, fewer clock hours of involvement, and less tortuosity of large blood vessels. Monitoring can be as frequent as every day in cases where premature infants are threatened with

retinal detachment from proliferative ROP. The frequency of monitoring will diminish with resolution of neovascularization.

EXAMPLE 4
DIABETIC RETINOPATHY

A. Preparation of Oligonucleotides

VEGF specific oligonucleotides are synthesized as described in EXAMPLE 2A above and prepared for administration as described in EXAMPLE 3A above. Doses to achieve intravitreal concentrations ranging from 0.1 - 100 μM are administered depending on the severity of the retinal/ocular neovascularization. The volume delivered is between 1 μl and 1 ml depending on the volume of the eye and whether vitreous has been previously removed as during a vitrectomy for diabetic eye disease.

B. Diabetic Patient Profile

The patient to be treated is a 30 year old African American male suffering for 25 years from juvenile-onset diabetes. The patient has bilateral proliferative retinopathy with sub-retinal hemorrhaging, cotton wool spots, and exudates. Upon fluorescein angiography, there are well defined areas of neovascularization bilaterally with areas of capillary drop-out.

C. Treatment

The patient is treated weekly with intraocular injections of oligonucleotides resuspended in the appropriate vehicle (BSS, Emanfour) at concentrations within the range of 0.1 - 100 μM.

The treatment may be supplemented with systemic delivery of oligonucleotide (i.e., intravenous, subcutaneous, or intramuscular) from 2 to 5 times per day to once a month, depending on the disease process and the biological half life of the oligonucleotides.

D. Monitoring of Progress

The patient's eyes are monitored as described above in EXAMPLE 2D. The eyes upon dilation are examined for regression of neovascularization with both a direct and an indirect ophthalmoscope to view the retina and fundus. A slit lamp exam is used in the case of anterior segment disease. Repeat injections are given as needed, based on the degree of inhibition of the neovascularization and the tolerance of the eye to injections. Short and long term follow-up check-ups are given to check for possible retinal detachment from the neovascular disease as well as from the injections.

EXAMPLE 5
AGE-RELATED MACULAR DEGENERATION

A. Preparation of Oligonucleotides

VEGF specific oligonucleotides are synthesized as described in EXAMPLE 2A above and prepared as described in EXAMPLE 3A above. Doses to achieve intravitreal concentrations ranging from 1 μl and 1 ml are administered depending on the severity of the retinal/ocular neovascularization. The volume delivered is between 1 μl and 1 ml depending on the volume of the eye and whether vitreous has been previously removed.

B. ARMD Patient Profile

The patient is a 50 year old Caucasian male suffering from the exudative form of age related macular degeneration. This patient has choroidal neovascularization which is apparent from fluorescein angiography. The disease is bilateral and the patient has a reduction in vision in each eye from 20/60 to 20/100.

C. Treatment

The patient is treated weekly with intraocular injections of oligonucleotide resuspended in the appropriate vehicle (BSS, Emanfour) at concentrations within the range of 0.1 to 100 μM. This treatment may be supplemented with systemic delivery of oligonucleotide (i.e., intravenous, subcutaneous, or intramuscular) from 2 to 5 times per day to once a month.

D. Monitoring of Progress

The eyes upon dilation are examined for regression of neovascularization with both a direct and an indirect ophthalmoscope to view the retina and fundus. Fluorescein angiography is used to check for the resolution of neovascularization. A slit lamp exam is used in the case of anterior segment disease. Repeat injections are given as needed, based on the degree of inhibition of the neovascularization and the tolerance of the eye to injections. Short and long term follow-up check-ups are given to check for possible retinal detachment from the neovascular disease as well as from the injections.

EXAMPLE 6
HUMAN CELL CULTURE

U373 human neuroblastoma cells were cultured in Dulbecco's modified Earls (DME) medium containing glucose (4500 mg/ml) and glutamate (2 mM) (Mediatech, Washington, DC) supplemented with penicillin/streptomycin (100 IU/MI/100 mcg/ml, Mediatech, Washington, DC). The cells were cultured at 37°C under 10% CO_2. The cells were plated in 96 well tissue culture dishes (Costar Corp., Cambridge, MA) and maintained as above. The cells were placed under anoxic conditions for 18-20 hours using an anaerobic chamber (BBL Gas Pak, Cockeysville, MD).

EXAMPLE 7
NORTHERN BLOTTING

In order to determine the level at which inhibition of VEGF expression occurs in cells in the presence of an oligonucleotide of the invention, Northern blotting was carried out. Human U373 cells cultured as described in EXAMPLE 6 above were plated in 100 mm tissue culture dishes and treated for 12 hours in the presence of 5 μg/ml lipofectin (Gibco-BRL, Gaithersburg, MD) as a lipid carrier with oligonucleotide H-3 (SEQ ID NO:6) (antisense oligonucleotide) and H-16 (SEQ ID NO:29) (sense oligonucleotide) at 0.05 μM, 0.5 μM, and 2.0 μM, respectively. The cells were refed after 12 to 15 hours with fresh media + oligonucleotide (minus lipofectin) and allowed to recover for 5 to 7 hours. The cells were placed in hypoxia for 18 to 20 hours total RNA was isolated using the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski and Sacchi (*Anal. Biochem.* (1987) **162**:156-159). Northern blotting was performed according to the methods of Sambrook et

al. (*Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Laboratory Press, NY) (1989) Vol. 1, pp. 7.38) or Arcellana-Panlilio et al. (*Meth. Enz.* (1993) **225**:303-328). All RNA signals were quantified on a Phosphorimager (BioRad, Hercules, CA) and normalized using the 36B4 cDNA probe (Laborda (1991) *Nucleic Acids Res.* **19**:3998).

EXAMPLE 8
ELISA VEGF PROTEIN STUDY

U373 neuroblastoma cells as described in EXAMPLE 6 above were plated in a 96 well tissue culture dish and treated overnight with varying concentrations of antisense oligonucleotides against human VEGF in the presence of 5 μg/ml lipofectin. The cells were refed after 12 to 15 hours with fresh media + oligonucleotide (no lipofectin) and allowed to recover for 5 to 7 hours. The dishes were placed under hypoxic conditions for 18 to 20 hours using an anaerobic chamber (Gas Pac, Cockeysville, MD). The media was analyzed using the antigen capture ELISA assay described above (approximately 36 hours post treatment). The human VEGF oligonucleotides used were H-3 (SEQ ID NO:6) (antisense, coding), H-16 (SEQ ID NO:29) (start site/coding, sense control), and a random control (R).

The culture medium from the cells described in EXAMPLE 5 was analyzed for VEGF protein as follows. 96-well plates (Maxizorb ELISA Nunc A/S, Camstrup, Denmark) were treated overnight at 4°C with 100 μl/well of the capture antibody, a monoclonal antibody against human VEGF (R&D Systems, Minneapolis, MN, 2.5 μg/ml in 1X PBS). The wells were washed three times with 1X PBS/0.05% Tween-20 (United States Biochemical, Cleveland, OH) using a

plate washer (Dynatech, Gurnsey Channel Islands). Non-specific binding sites in the wells were blocked by adding 2% normal human serum (100 μl) and incubating the plate at 37°C for 2 hours. This blocking solution was removed and 200 μl conditioned medium containing human VEGF added to each well and incubated at 37°C for 2 to 3 hours. The plates were washed as described above. 100 μl of the primary antibody (618/619, 2 μg/ml in normal human serum) was added to each well and incubated at 37°C for 1 to 2 hours. The secondary antibody was an affinity purified rabbit anti- human VEGF polyclonal). The plates were washed as described above. 100 μl of the detection antibody, a horse radish peroxidase-labelled goat anti-mouse IgG monoclonal antibody (1:10,000, Vector Laboratories, Burlinggame, CA), was added to each well and incubated at 37°C for 1 hour. The plates were washed as described above. The wells were developed using the TMB microwell peroxidase developing system (Kirkegaard and Perry, Gaithersburg, MD), and quantified at 450 nm using a Ceres 900 plate reader (Bio-Tek Instruments, Inc., Winooski, Vermont). The linear range of this assay is between 2 ng and 0.01 ng human VEGF. Representative results are shown in FIG. 3.

EXAMPLE 9
BIOACTIVITY ASSAYS

Bioactivity can be determined by the Miles vessel permeability assay (Miles and Miles (1952) *J. Physiol. (Lond.)* **118**:228). Briefly, Hartley guinea pigs (800 g) are shaved and depilated and injected intravenously with 1.0 ml of normal saline containing 0.5 g of Evans Blue dye per 100 ml. Subcutaneous injections (250 μl) of serum-free medium containing unknown quantities of VEGF are

performed. Positive (purified VEGF) and negative controls (normal saline) are also included in the experiment. Twenty minutes post-injection, the animals are sacrificed and the test and control sites are cut out and quantitated for extravasation of Evans Blue dye. The limit of detection for this assay is 500 pM.

Endothelial cell mitogenicity can also manifest bioactivity. In this method, human umbilical vein endothelial (HUVEC) are grown and maintained using the Biocoat endothelial cell growth environment (Collaborative Biomedical Products, Bedford, MA). 1 x 10^4 cells are then plated in duplicate on 35 mM tissue culture dishes in 1.4 ml E-STIM medium (Collaborative Biomedical Products, Bedford, MA) plus 5% heat-inactivated fetal bovine serum. Following cell attachment (about 4 hours), two dishes of cells are trypsinized, counted, and used for a starting cell number. Test samples containing unknown amounts of VEGF are then added in duplicate to the remaining dishes at day 0 and at day 2. Controls consisting of purified VEGF (positive) and PBS (negative) are also used. On day 4, the dishes of cells are trypsinized, counted and compared to the starting cell number. The limit of detection for this assay is 10 pM.

The intracellular calcium release assay is also used to monitor bioactivity (see, e.g., Brock and Capasso (1988) *J. Cell. Physiol.* **136**:54). Human umbilical vein endothelial cells (HUVEC) are maintained in EGM-UV medium. Cells are removed from the plate by means of EDTA and collagenase. The calcium-sensitive dye, Fura-2 (Molecular Probes, Eugene, OR), is used to monitor changes in the concentration of intracellular calcium. In brief, medium

containing an unknown concentration of VEGF is added to an aliquot of suspended HUVEC, pre-loaded with Fura-2. Changes in fluorescence representing changes in intracellular calcium release are measured using a Hitachi 2000 F fluorometer. Positive (histamine, thrombin) and negative (EGTA) controls are also analyzed. This method is extremely sensitive and has a limit of detection of 0.2 pM.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Hybridon, Inc.
Children's Medical Center Corp.

(ii) TITLE OF INVENTION: Inhibition of Neovascularization Using VEGF-Specific Oligonucleotides

(iii) NUMBER OF SEQUENCES: 53

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 200 State Street
(C) CITY: Boston
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE:

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kerner, Ann-Louise
(B) REGISTRATION NUMBER: 33,523
(C) REFERENCE/DOCKET NUMBER: HYZ-031PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617-330-1300
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGCCGGGCCG CCAGCACACT 20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGCCGGGCCG CCAGCACACU 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGCCGCCAGC ACACT 15

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCTCGCGCCG GGCCGCCAGC ACACT 25

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CAAGACAGCA GAAAGTTCAT 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CACCCAAGAC AGCAGAAAG 19

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CACCCAAGAC AGCAG 15

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCAATGCACC CAAGACAGCA GAAAG 25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ACGCACACAG AACAAGACG 19

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAGTTCATGG TTTCGGAGGC 20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTTCATGGTT TCGGAGGCCC 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTGCAGCCTG GGACCACTTG 20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CGCCTCGGCT TGTCACATCT 20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTTCCTCCTG CCCGGCTCAC 20

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CUUCCUCCUG CCCGGCUCAC 20

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTTCCTCCTG CCCGG

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGCTCCTTCC TCCTGCCCGG CTCAC

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTCTCCTCTT CCTTCATTTC

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GTCTCCTCTT CCTTC

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GCAGAGTCTC CTCTTCCTTC ATTTC

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CGGACCCAAA GTGCTCTGCG

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CCAAAGTGCT CTGCG

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CCCTCCGGAC CCAAAGTGCT CTGCG

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GGGCACGACC GCTTACCTTG

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GGGACCACTG AGGACAGAAA 20

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CACCACTGCA TGAGAGGCGA 20

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TCCCAAAGAT GCCCACCTGC 20

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGCATAATCT GGAAAGGAAG 20

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ACTTTCTGCT GTCTTGGGTG 20

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CATGGTTTCG GAGGCCCGAC 20

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA/RNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CAUGGTTUCG GAGGCCCGAC 20

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TTCATGGTTT CGGAGGCCCG 20

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GACCGCTTAC CTTGGCATGG 20

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CCTGGGACCA CTGAGGACAG 20

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GGGACTCACC TTCGTGATGA 20

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GAACTTCACC ACTGCATGAG 20

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

TCCCAAAGAT GCCCACCTGC 20

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GCATAATCTG GAAAGGAAGG 20

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

ACATCCTCAC CTGCATTCAC 20

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

ACATCCUCAC CTGCAUUCAC 20

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

TTTCTTTGGT CTGCAATGGG 20

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GGCCACTTAC TTTTCTTGTC 20

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CACAGGGACT GGAAAATAAA 20

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

GGGAACCAAC CTGCAAGTAC 20

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GTCACATCTG AGGGAAATGG 20

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CAGCCTGGCT CACCGCCTTG G 21

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

TCGCGCTCCC TCTCTCCGGC 20

(2) INFORMATION FOR SEQ ID NO:48

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CATGGTTTCG GAGGGCGTC 19

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

TCCGAAACCA TGAACTTTCT G 21

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

GGTTTCGGAG GCCCGACCG 19

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

CAAGAGAGCA GAAAGTTCAT 20

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

CACCCAAGAG AGCAGAAACT 20

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

TCGTGGGTGC AGCCTGGGAC 20

What is claimed is:

1. A method of reducing neovascularization in a tissue comprising the step of administering to the tissue an amount of a synthetic oligonucleotide specific for vascular endothelial growth factor nucleic acid which is effective in inhibiting the expression of vascular endothelial growth factor.

2. The method of claim 1 wherein the oligonucleotide is modified.

3. The method of claim 2 wherein the modified oligonucleotide has at least one internucleotide linkage selected from the group consisting of alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters.

4. The method of claim 3 wherein the modified oligonucleotide has at least one phosphorothioate internucleotide linkage.

5. The method of claim 1 wherein the oligonucleotide comprises a ribonucleotide, a deoxyribonucleotide, or a combination thereof.

6. The method of claim 5 wherein the oligonucleotide comprises at least one 2'-O-alkylated ribonucleotide.

7. The method of claim 1 wherein the oligonucleotide is from about 14 to about 28 nucleotides in length.

8. The method of claim 7 wherein the oligonucleotide is about 15 to about 25 nucleotides in length.

9. A pharmaceutical composition capable of inhibiting neovascularization comprising a synthetic oligonucleotide which specifically inhibits the expression of vascular endothelial growth factor and a physiologically acceptable carrier.

10. A method of treating retinopathy of prematurity comprising the step of administering to a subject afflicted with retinopathy of prematurity a therapeutic amount of an oligonucleotide specific for vascular endothelial growth factor nucleic acid and effective in inhibiting the expression of vascular endothelial growth factor in the retina.

11. The method of claim 10 wherein the oligonucleotide is administered locally or systemically.

12. A method of treating diabetic retinopathy comprising the step of administering to a subject afflicted with diabetic retinopathy a therapeutic amount of an oligonucleotide specific for vascular endothelial growth factor nucleic acid and effective in inhibiting the expression of vascular endothelial growth factor in the retina.

13. The method of claim 12 wherein the oligonucleotide is administered locally or systemically.

14. A method of treating age-related macular degeneration comprising the step of administering to a subject afflicted with age-related macular degeneration a therapeutic amount of an oligonucleotide specific for vascular endothelial growth factor nucleic acid and effective in inhibiting the expression of vascular endothelial growth factor in the retina.

15. The method of claim 14 wherein the oligonucleotide is administered locally or systemically.

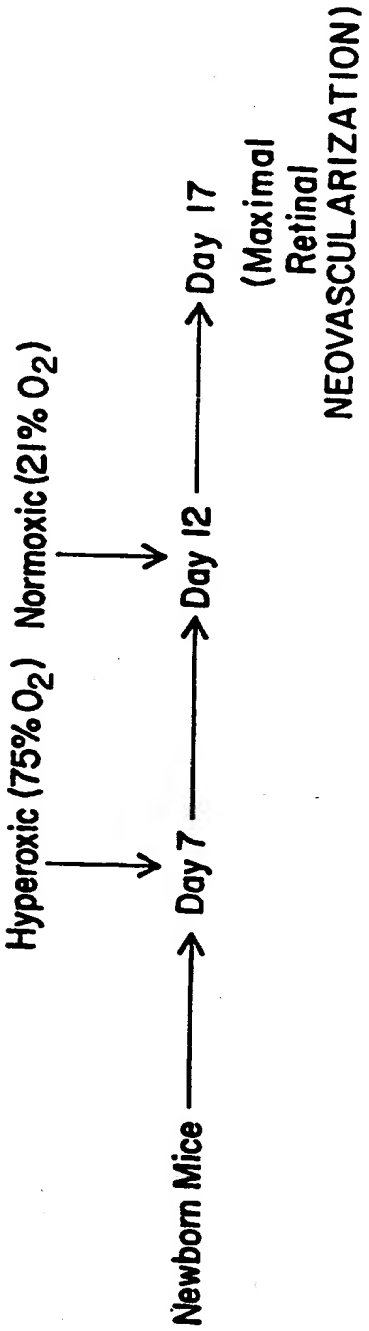
Newborn Mice
Hyperoxic (75% O_2)
Day 7
Normoxic (21% O_2)
Day 12
Day 17
(Maximal Retinal NEOVASCULARIZATION)

FIG. 1

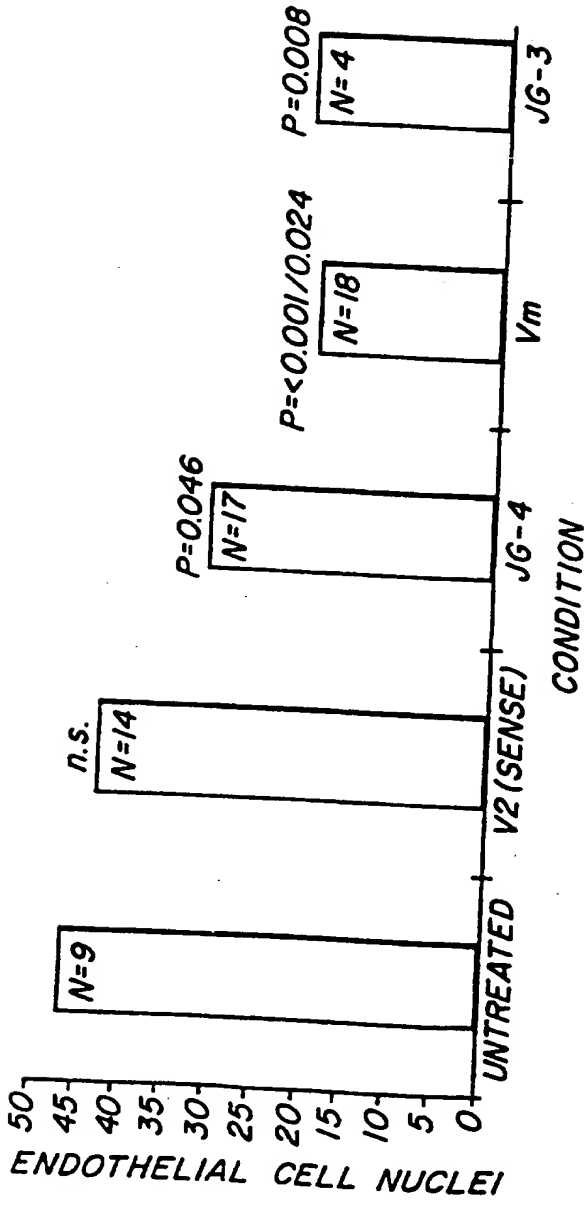

FIG. 2

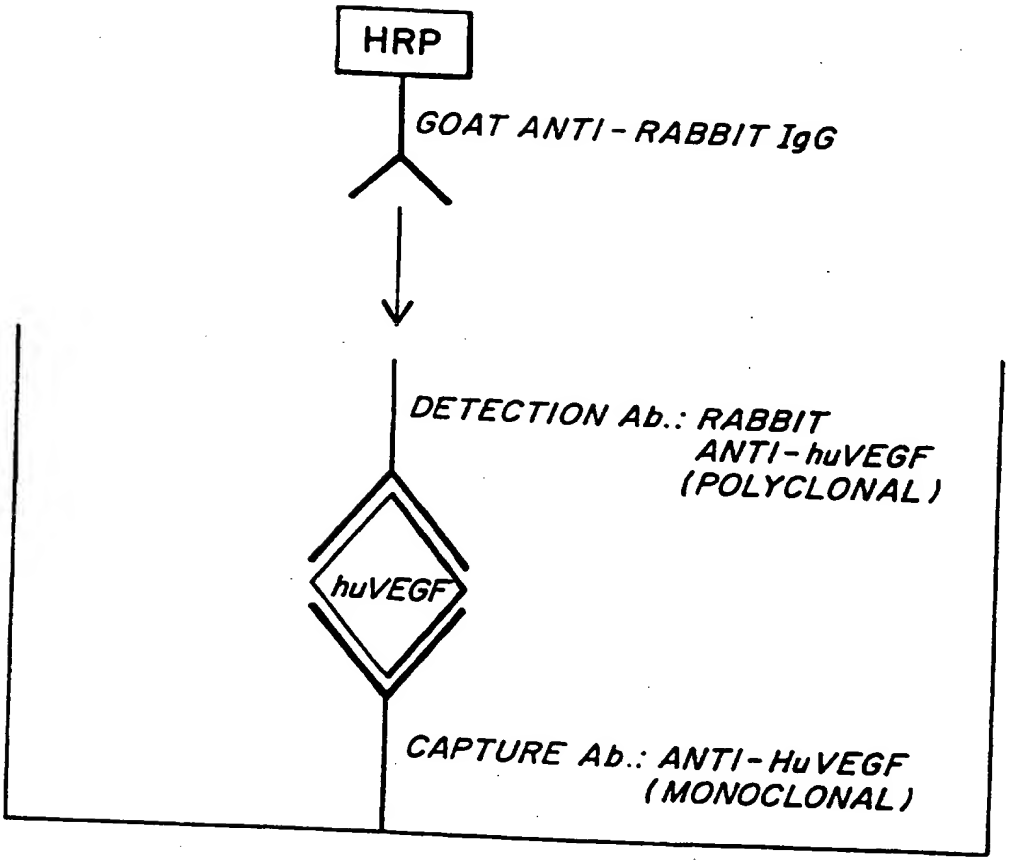
HRP
GOAT ANTI-RABBIT IgG
DETECTION Ab.: RABBIT ANTI-huVEGF (POLYCLONAL)
huVEGF
CAPTURE Ab.: ANTI-HuVEGF (MONOCLONAL)

FIG. 3

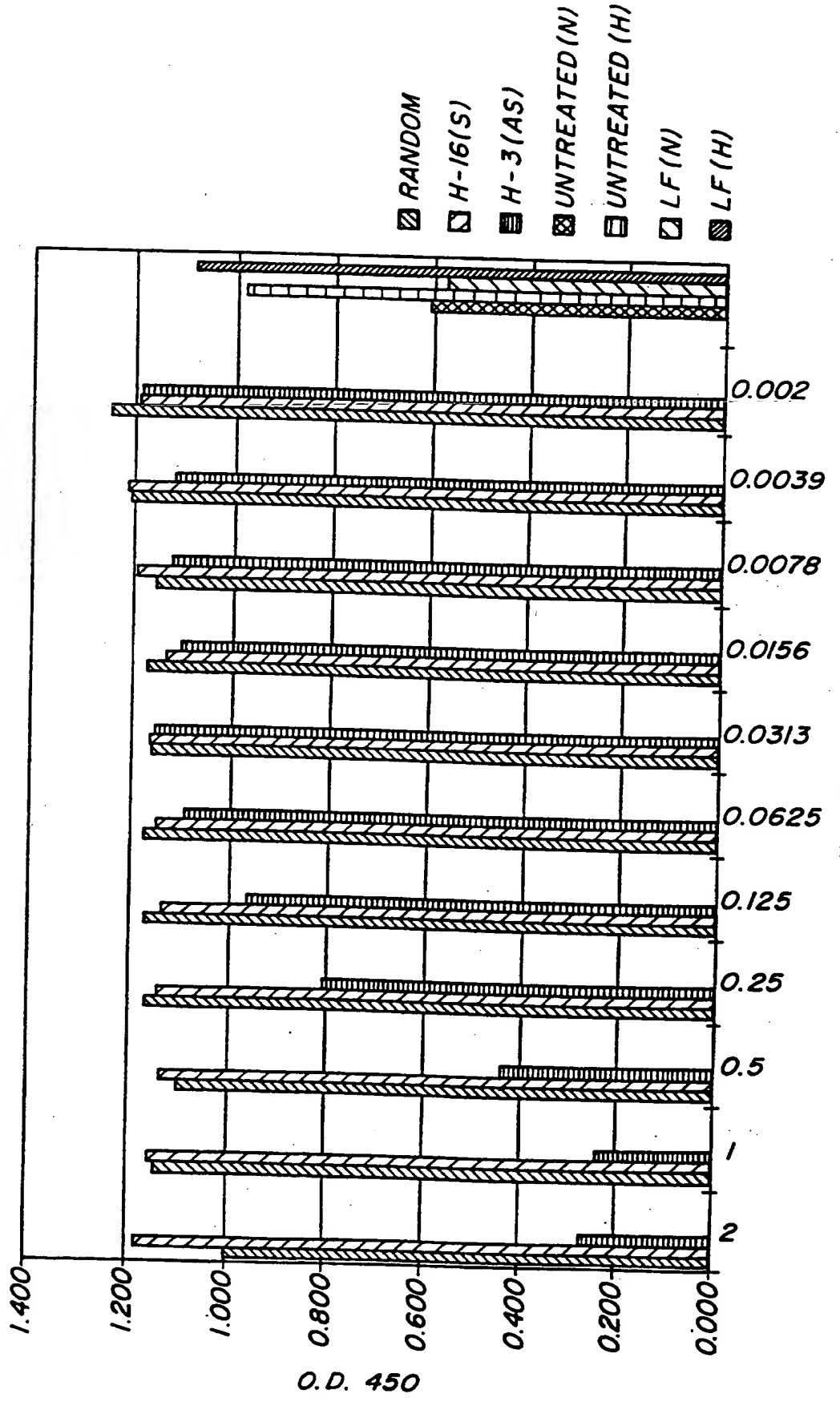

FIG. 4

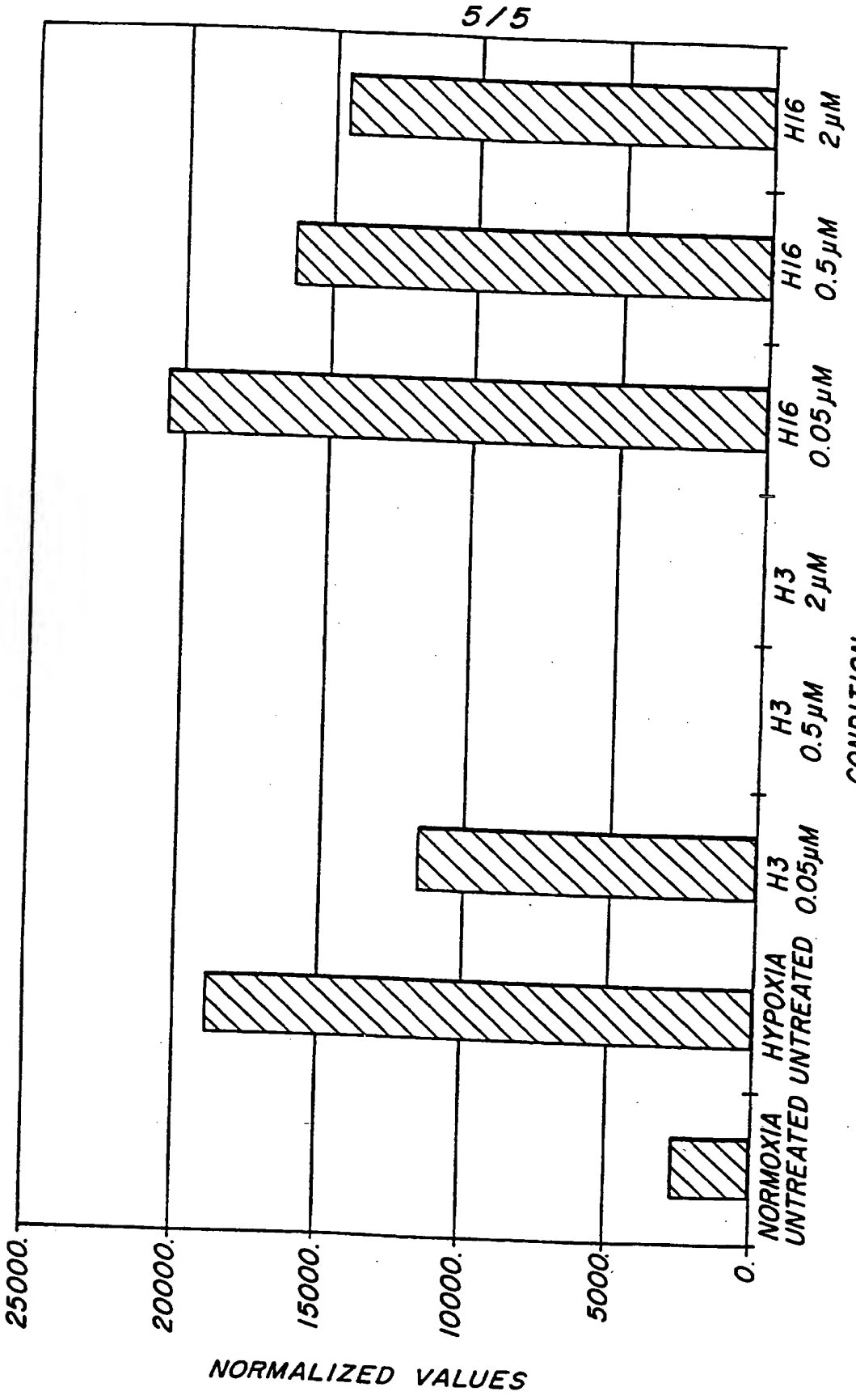
25000.
20000.
15000.
10000.
5000.
0.
NORMALIZED VALUES
NORMOXIA UNTREATED
HYPOXIA UNTREATED
H3 0.05μM
H3 0.5μM
H3 2μM
H16 0.05μM
H16 0.5μM
H16 2μM
CONDITION

FIG. 5

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

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(54) Title: INHIBITION OF NEOVASCULARIZATION USING VEGF-SPECIFIC OLIGONUCLEOTIDES

(57) Abstract

Disclosed are methods of reducing neovascularization and of treating various disorders associated with neovascularization. These methods include administering to a tissue or subject a synthetic oligonucleotide specific for vascular endothelial growth factor nucleic acid effective in inhibiting the expression of vascular endothelial growth factor.

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 96/01189

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 A61K31/70 C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,X	ANTISENSE RES.DEV. 5 (SPRING 95); PAGE 91; ABSTRACT OP-18 , XP002008710 NOMURA, M. ET AL.: "Expression and function of VEGF gene in hypoxia-induced proliferation of vascular cells"	1-5,9
Y	see abstract & 1ST INT.ANTISENSE CONF.JAPAN, 4 - 7 December 1994, ---	1-13
Y	THE NEW ENGLAND JOURNAL OF MEDICINE, vol. 331, 1 December 1994, pages 1480-1487, XP000574787 AIELLO, L. ET AL.: "Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders" cited in the application see the whole document --- -/--	1,9,12, 13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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1

Date of the actual completion of the international search	Date of mailing of the international search report
18 July 1996	25. 07. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Andres, S

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL REVIEWS, vol. 90, no. 4, 1 June 1990, pages 543-584, XP000141412 UHLMANN, E. ET AL.: "ANTISENSE OLIGONUCLEOTIDES: A NEW THERAPEUTIC PRINCIPLE" cited in the application see the whole document ---	2-8
P,Y	INVESTIGATIVE OPHTHALMOLOGY & VISUAL SCIENCE 36 (4). S871. ABSTRACT 3990, 15 March 1995, XP002008711 PIERCE, E. ET AL.: "Regulation of retinal vascular endothelial growth factor (VEGF/VPF) levels by hyperoxia and hypoxia: a possible etiology for ROP" see abstract & ANNUAL MEETING OF THE INVESTIGATIVE OPHTHALMOLOGY AND VISUAL SCIENCE, FORT LAUDERDALE, FLORIDA, USA, MAY 14-19, 1995, ---	10,11
A	ANTISENSE RES.DEV. 5 (SPRING 95); PAGES 87-8; ABSTRACT OP-10 , XP002006442 UCHIDA, K. ET AL.: "Selection of antisense oligodeoxyribonucleotides that inhibit VEGF/VPF expression in a cell-free system" cited in the application see abstract & 1ST INT.ANTISENSE CONF.JAPAN , 4 - 7 December 1994, ---	1-15
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 193, 15 June 1993, ORLANDO, FL US, pages 631-638, XP002008712 ADAMIS, A. ET AL.: "Synthesis and secretion of vascular permeability factor/vascular endothelial growth factor by human retinal pigment epithelial cells" cited in the application ---	
P,X	WO,A,95 04142 (HYBRIDON INC ;ROBINSON GREGORY S (US)) 9 February 1995 see the whole document ---	1-9,12, 13

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	INVESTIGATIVE OPHTHALMOLOGY & VISUAL SCIENCE 36 (4). S871, ABSTRACT 3992, 15 March 1995, XP002008713 SMITH, L. ET AL.: "Inhibition of proliferative retinopathy using antisense phosphorothioate oligonucleotides against vascular endothelial growth factor (VEGF - VPF)." see abstract & ANNUAL MEETING OF THE INVESTIGATIVE OPHTHALMOLOGY AND VISUAL SCIENCE, FORT LAUDERDALE, FLORIDA, USA, MAY 14-19, 1995, ---	1-4,9
T	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, 93/10 (4851-4856), May 1996, XP002008714 ROBINSON, G. ET AL.: "Oligodeoxynucleotides inhibit retinal neovascularization in a murine model of proliferative retinopathy." see the whole document -----	1

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01189

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-8,10-15 are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. claims 1-9 Method for reducing neovascularization in a tissue
2. claims 10-11 Method for treating retinopathy of prematurity
3. claims 12-13 Method of treating diabetic retinopathy
4. claims 14-15 Method of treating age-related macular degeneration

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [X] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/01189

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9504142	09-02-95	AU-B-	7516894	28-02-95
		CA-A-	2167680	09-02-95
		EP-A-	0711343	15-05-96
		FI-A-	960374	25-03-96
		NO-A-	960303	13-03-96

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